

AD _____

Award Number: DAMD17-03-1-0392

TITLE: The Role of Notch Signaling Pathway in Breast Cancer Pathogenesis

PRINCIPAL INVESTIGATOR: Annapoorni Rangarajan, Ph.D.

CONTRACTING ORGANIZATION: Whitehead Institute for Biomedical Research
Cambridge, Massachusetts 02142

REPORT DATE: July 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20050121 006

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)		2. REPORT DATE July 2004	3. REPORT TYPE AND DATES COVERED Annual Summary (30 Jun 2003 - 29 Jun 2004)	
4. TITLE AND SUBTITLE The Role of Notch Signaling Pathway in Breast Cancer Pathogenesis			5. FUNDING NUMBERS DAMD17-03-1-0392	
6. AUTHOR(S) Annapoorni Rangarajan, Ph.D.				
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Whitehead Institute for Biomedical Research Cambridge, Massachusetts 02142 E-Mail: anur@wi.mit.edu			8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER	
11. SUPPLEMENTARY NOTES				
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited			12b. DISTRIBUTION CODE	
13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Constitutively active forms of Notch receptors have been shown to have oncogenic potential in several cell-types. Aberrant expression of Notch signaling pathway components has been detected in several human tumors, including breast cancer. Since Ras signaling is also activated in several breast cancers, I propose to study the potential interactions between Notch and Ras signaling in breast carcinogenesis. Initial observations revealed that co-expression of a constitutively active form of Notch1 in immortalized breast epithelial HMLE cells expressing low levels of oncogenic Ras rendered them fully transformed. These cells generated soft agar colonies in vitro, and tumors when injected subcutaneously in nude mice. Further characterization of the Notch-Ras pathway interaction revealed that nuclear localization of Notch1 is essential for this cooperation. Dissection of Ras-pathways using the Ras-effector loop mutants revealed that compared to G37 and C40, that activate Ral-GEF and PI3K, respectively, the S35 mutant, which preferentially activates Raf/MAPK pathway, formed efficient colonies with activated Notch1. Interestingly, I found that expression of activated Notch1 rendered the HMLE-low Ras cells highly spindle and fibroblastic - characteristics of epithelial-to-mesenchyme transition (EMT) associated with tumor invasion and metastasis. Very recent reports have indeed demonstrated a role for Notch signaling in EMT associated with normal development and cancers. Accordingly, I investigated the role of Notch signaling in the EMT phenotype of breast cancer cells. I found that inhibition of Notch signaling, using presenilin inhibitor, caused the highly metastatic SUM1315 breast cancer cells to revert to an epithelial phenotype, thereby suggesting that Notch signaling may play an important role in mediating/maintaining the EMT phenotype of breast cancer cells. To further investigate the role of Notch signaling in breast cancers, I have generated an siRNA against Jagged1, a Notch-ligand. In preliminary experiments this siRNA suppressed Jagged1-expression by greater than 90-fold.				
14. SUBJECT TERMS notch, Ras, signaling, transformation, tumorigenesis			15. NUMBER OF PAGES 9	
			16. PRICE CODE	
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited	

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusions.....	7
References.....	7
Appendices.....	none

The role of Notch Signaling Pathway in Breast Cancer Pathogenesis

Introduction

Notch proteins belong to a family of four receptors, Notch 1-4, which are activated upon binding to ligands of the Jagged/Delta family. Ligand binding induces a series of cleavage in the Notch protein, thereby causing the release of its cytoplasmic region in to the nuclei, where it functions as a transcriptional activator. Truncated and constitutively active forms of all four Notch proteins have been shown to have oncogenic functions in several murine cell-types. In an MMTV insertional mutagenesis model, integration of the integration of MMTV at the *int3* locus caused mammary tumorigenesis through the activation of the *int3/Notch4* gene. Importantly, aberrant expression of Notch pathway components has been detected in human breast cancers (1,2). Taken together, these observations implicate a role for Notch signaling in human breast cancers; however, a clear functional involvement is yet to be demonstrated.

I propose to study the role of Notch signaling using the genetically defined immortalized and transformed human breast cell system developed in our lab (3). In this model normal human mammary epithelial cells (HMECs) were immortalized and transformed by introducing a defined set of genetic elements. Thus, introduction of the simian virus 40 early region (SV40-ER) and the catalytic subunit of human telomerase enzyme rendered the HMECs immortalized (these immortalized HMECs are referred to as HMLE cells). Subsequent expression of supra-physiologic levels of oncogenic Ras rendered the HMLE cells fully transformed. Thus, this model provides a unique, genetically well characterized system in which one can study interaction of signaling pathways involved in breast cancer pathogenesis, without the interference of unknown genetic alterations sustained by breast cancer-derived cell lines normally used for such studies.

However, in the model system described above, low or physiologically relevant levels of oncogenic Ras failed to transform HMLE cells. Similarly, overexpression of ErbB2, a receptor tyrosine kinase upstream of Ras normally found overexpressed in many breast cancers, also failed to transform HMLE cells. These observations suggest that in order for physiologically relevant levels of Ras signaling to transform immortalized breast cells, cooperation of additional signaling pathways is essential. I hypothesize that the Notch proteins are a likely candidate for such cooperation.

Results:

A) Characterization of Notch-Ras pathway cooperation in immortalized breast epithelial cell transformation

1) Expression of Notch1^{IC} mediates transformation of HMLER cells

Over expression of constitutively active, intracellular domain of Notch1 (hereafter termed Notch1^{IC}) in immortalized HMLE breast cells resulted in cell death. Therefore, the transformation related phenotype of cells expressing Notch1^{IC} alone could not be studied. However, introduction of Notch1^{IC} into HMLE cells expressing low levels of Ras (hereafter termed HMLERs) allowed for selection of cells expressing Notch1^{IC}. Figure

1A shows a Western blot analysis revealing high levels of expression of Notch1^{IC} in HMLER cells. In order to characterize the transformation related properties of Notch1^{IC}-expressing HMLER cells I undertook soft agar colony formation analysis. While HMLER cells failed to generate colonies in soft agar, Notch1^{IC}-expressing HMLER cells generated efficient colonies (Fig 1B). This suggested that signals emanated by the activation of Notch and Ras can indeed cooperate to mediate transformation of immortalized breast epithelial cells.

To further determine whether this Notch-Ras cooperation suffices to give rise to tumors *in vivo*, I injected 2x10⁶ Notch1^{IC}-expressing HMLER cells and parental HMLER cells, admixed with Matrigel, into the subcutaneous space of immuno-compromised nude mice. As shown in Fig 1C, the Notch1^{IC}-expressing cells generated tumors while the parental HMLER cells failed to. Taken together, these observations indicated that Notch mediates transformation of breast cells by cooperating with Ras signaling

2) The Raf/MAPK pathway downstream of Ras cooperates with Notch1^{IC} in transforming HMLE cells

Upon activation Ras signals through multiple downstream effector pathways. The three major pathways downstream of Ras that have been shown to play an important role in transformation involve the PI3K, Raf/MAPK, and Ral-GEFs. In order to determine which of these Ras pathways is actually involved in cooperating with Notch1^{IC} to transform HMLER cells, I employed the effector-loop mutants of oncogenic Ras – S35 G37, and C40 (4). These mutants signal preferentially through only one pathway. Thus, S35 preferentially activates Raf, C40 activates PI3K, and G-37 activates Ral-GEFs. Each of the three Ras mutants was individually expressed in HMLE cells. Thereafter, Notch1^{IC} was expressed, and drug resistant colonies were selected. The resultant cells were subjected to soft-agar analysis. I found that HMLE cells expressing S35 and Notch1^{IC} generated colonies with maximum efficiency, while cells expressing C40 or G37 and Notch1^{IC} generated far fewer colonies. These results suggested that the Raf/MAPK pathway is the main Ras-downstream pathway that is involved in cooperating with Notch1^{IC} in transforming immortalized breast epithelial cells.

3) Nuclear localization of Notch1^{IC} is essential for its transforming properties

Following ligand binding, Notch is proteolytically cleaved and its intracellular domain translocates to the nucleus, where it has been shown to function as a transactivator by replacing CBF1. In order to determine whether nuclear localization of Notch1^{IC} is required for its transforming functions, I introduced a nuclear localization defective mutant of Notch1^{IC} (5) into HMLER cells and subjected the resultant cells to soft agar analysis. The results revealed that the nuclear localization defective mutant of Notch1^{IC} failed to transform HMLER cells. This suggested that the nuclear localization of Notch1^{IC} is essential for its cooperation with Ras in transforming HMLER cells.

B) Assess cooperation between activated Notch and ErbB2 signaling in breast cancer

Breast cancers typically do not harbor oncogenic Ras mutations; nevertheless,

Ras signaling is activated in many breast cancers, mainly due to alteration or overexpression of upstream receptor tyrosine kinase. For example, ErbB2 is altered or overexpressed in ~30% of all breast cancers; however we found that ErbB2 overexpression in HMLE cells failed to transform them. Accordingly, I determined whether Notch1^{IC} could transform wild type ErbB2-expressing HMLE cells. However, Notch1^{IC}-expressing HMLE-ErbB2 cells failed to generate colonies in soft agar. One possible reason for this could be that overexpression of ErbB2 only weakly activates Ras. Consequently, I am currently overexpressing a constitutively active allele of ErbB2 (6), which has been shown to have oncogenic potential, in HMLE cells. Thereafter, I will determine whether Notch1^{IC} can transform these cells.

C) Determine whether Notch Signaling is required for the transformed phenotype of breast cancer cells

I observed that expression of Notch1^{IC} into HMLER cells rendered these cells very spindly and fibroblastic – characteristics of an epithelial to mesenchymal transition (EMT) associated with tumor invasion and metastasis. Very recent reports have indeed demonstrated a role for Notch signaling in EMT associated with normal development and cancers (7). Accordingly, I investigated the role of Notch signaling in the EMT phenotype of breast cancer cells.

Presenilin mediated proteolytic cleavage of Notch has been shown to be necessary for the functioning of wild type Notch receptors (8); accordingly, chemical inhibitors of presenilin have been shown to impair Notch signaling (9). Therefore, I used DAPT, a chemical inhibitor of presenilin, to inhibit Notch signaling in a highly metastatic breast cancer cell line, SUM1315. Interestingly, when SUM1315 breast cancer cells were grown in the presence of DAPT, they lost their fibroblastic appearance and reverted to an epithelial phenotype (Fig 2A). This data provides strong evidence supporting an important role for Notch signaling in mediating and/or maintaining the EMT phenotype of breast cancer cells. I propose to further characterize this phenomenon by undertaking Western blot analysis for markers of EMT.

To further investigate the role of Notch signaling in breast cancers, I propose to inactivate Notch signaling in breast cancer cell lines using siRNA approach. I have found that Jagged1, a Notch-ligand, is over expressed in several breast cancer cell lines, whereas primary or immortalized breast epithelial cells lack its expression (Fig. 2B). This suggests that Jagged1 may be the prime ligand which signals to Notch proteins in breast cancer cells. Therefore, I will attempt to suppress the expression of Jagged1 in breast cancer cells (such as SUM1315, SKBR3 which overexpress ErbB2 and MDA-MB-231 cells which harbor an oncogenic mutant of Ras) using an siRNA approach and then determine its effect on their transformation potential. I will examine the effect of Jagged1 suppression in tumor initiation, maintenance, and ability to seed metastasis.

Towards this end, I have generated siRNAs against Jagged1 using the pLKO1 lenti-viral siRNA vector developed in our laboratory. In preliminary experiments I have found that one such siRNA is capable of suppressing Jagged1 expression by >90-fold (lane 5, Fig.

2C). This result is very encouraging, and I am currently infecting several breast cancer cell lines with this siRNA.

Key Research Accomplishments:

- 1) Generation of HMLER cells expressing Notch1^{IC} and characterization of their transformed phenotype.
- 2) Characterization of Notch-Ras pathway interaction in HMLE cells using a variety of mutants.
- 3) Generation and characterization of Notch1^{IC}-expressing HMLE-ErbB2 cells.
- 4) Identification of a role for Notch signaling in EMT (epithelial-to-mesenchyme transition) involved in tumor invasion and metastasis.
- 5) RT-PCR analysis of Notch and ligand expression in breast cancer cell lines
- 6) Generation and characterization of an siRNA against a Notch receptor, Jagged1, which is commonly overexpressed in breast cancer cells.

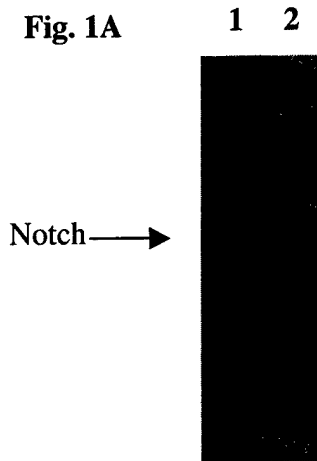
Reportable outcomes

- 1) Expression of Notch1^{IC} in HMLER cells resulted in soft agar colony formation and tumor initiation in mice, indicating a strong cooperation between Notch and Ras pathways in breast cell transformation.
- 2) Nuclear localization of Notch1^{IC} is essential for this Notch-Ras cooperation
- 3) The Raf/MAPK pathway downstream of Ras plays a major role in the Notch-Ras cooperation
- 4) Inhibition of Notch signaling reverts highly metastatic (EMT phenotype) breast cancer cell line, SUM1315, to an epithelial phenotype.
- 5) A Notch-ligand, Jagged1, is overexpressed in several breast cancer cell lines compared to normal or immortalized breast cells.
- 6) The siRNA generated against Jagged1 mediates >90-fold suppression of Jagged1

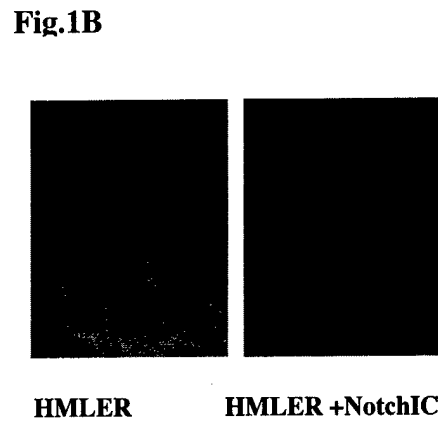
Conclusion: The results obtained thus far strongly suggest that Notch proteins may function as key mediators of breast cancer pathogenesis. The cooperation of Notch with Ras in transforming immortalized breast cells gives a mechanistic insight into the role of Notch signaling in this process. The requirement for Notch signaling in maintaining the EMT phenotype of SUM1315 cells is indicative of a role for Notch proteins in breast cancer invasion and metastasis. This experiment, however, needs to be repeated with several breast cancer cells. Taken together these results strongly suggest a functional involvement of Notch signaling in breast cancer pathogenesis. A clear demonstration of the requirement of Notch signaling in breast carcinogenesis will enable the targeting of Notch pathway as a new therapeutic approach against breast cancers.

References

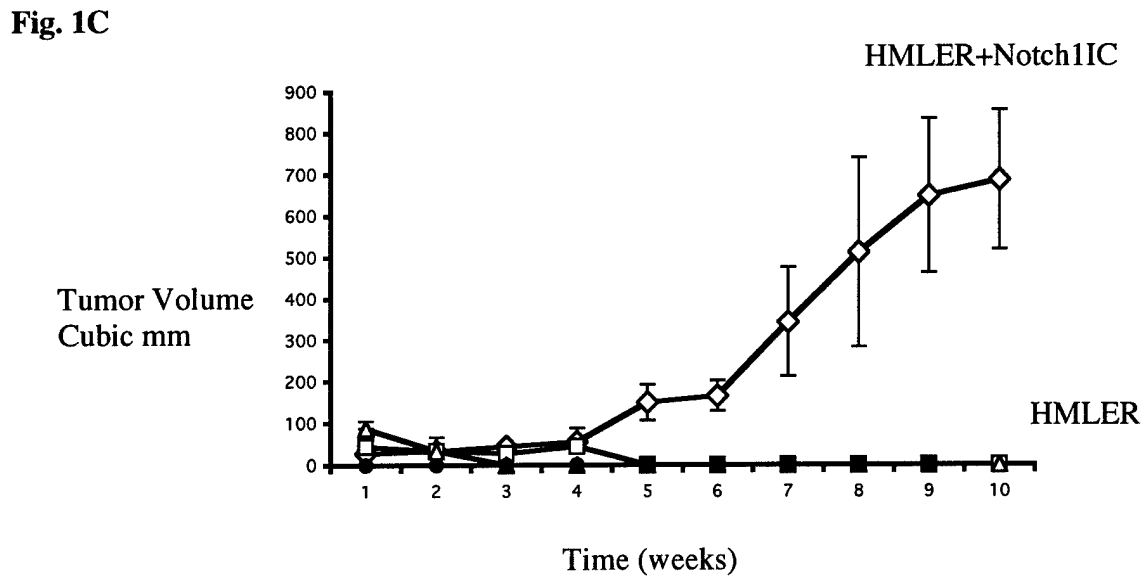
- 1) Miele L and Osborne B, *J Cell Physiol*, **181**, 393-401 (1999)
- 2) Weng AP and Aster JC, *Curr Opin Genet Dev*, **14** (1):48-54 (2004)
- 3) Elenbaas B et al., *Genes Dev*, **15**, 50-65 (2001)
- 4) Rodriguez-Viciana P. et al., *Cell*, **89**, 457-57 (1997)
- 5) Jeffries S, et al., *Mol Cell Biol*, **20**, 3928-41 (2000)
- 6) Seton-Rogers SE et al., *Proc Natl Acad Sci U S A*. **3**;101(5):1257-62 (2004)
- 7) Grego-Bessa J et al., *Cell Cycle*, **3**(6):718-21 (2004)
- 8) Struhl G et al., *Proc Natl Acad Sci U S A*. **2**;98(1):229-34 (2000)
- 9) Berezovska O, et al., *Neurochem* **75**, 583-93 (2000)



Expression of Notch1 in HMLER (1) and 293T (2) cells



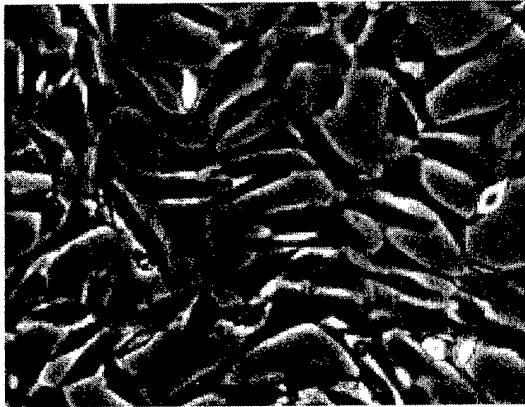
Soft-agar colony formation assay



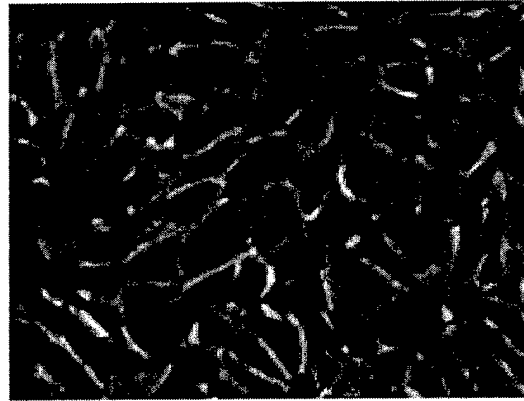
Tumor growth curves formed by HMLE, HMLER, and Notch1IC-expressing HMLER cells.

Fig 2A

DMSO

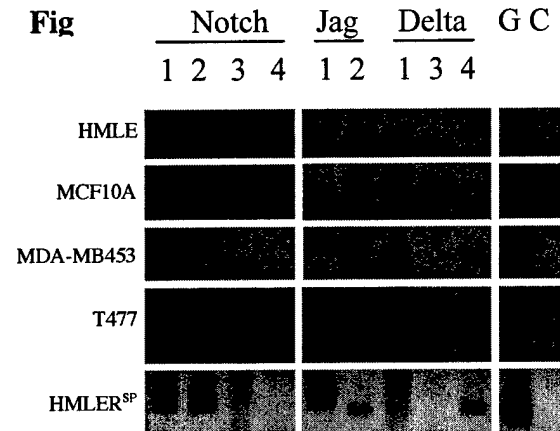


DAPT



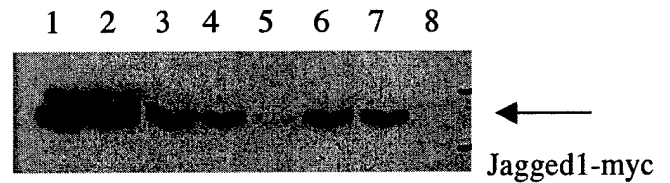
SUM1315 cells grown in the presence of control DMSO or 5uM DAPT dissolved in DMSO.

Fig 2B



RT PCR analysis for Notch and ligand expression in immortalized and breast cancer cells. G refers to GAPDH PCR; C refers to minus RT control.

Fig 2C



Western blot analysis for myc-tagged Jagged1 using anti-myc Ab. 293T cells were transiently cotransfected with a Jagged1-myc expression Vector and various siRNA constructs designed against Jagged1-- Lane1, control vector; Lanes 2-3, different siRNA constructs, Lane8, no DNA control.